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USARMY MEDICAL RESEARCH LABORATORY

FORT KNOX, KENTUCKY 40121

REPORT NO. 897

MODIFIED SICKLEDEX TUBE TEST: A SPECIFIC TEST FOR S HEMOGLOBIN

(Progress Report)

by

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17 September 1970

UNITED STATES ARMY
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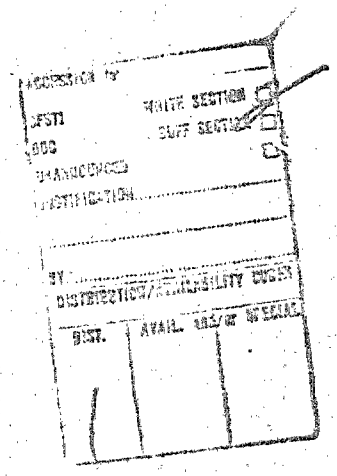

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ABSTRACT

MODIFIED SICKLEDEX TUBE TEST:
A SPECIFIC TEST FOR S HEMOGLOBIN

OBJECTIVE

To establish the principle of the Sickledex test for S hemoglobin which was not disclosed by the developers of the test. This screening test was to be modified if possible to develop a specific test for S hemoglobin suitable for testing large human populations.

METHODS

The Sickledex kit and reagents as supplied by the manufacturer were used throughout the study. Stock solutions of urea were used.

RESULTS

The Sickledex test is a screening method for the detection of S hemoglobin. However, the principle of this test has never been disclosed by the developers of the test. On the basis of analytical studies conducted in the Blodgett Memorial Hospital laboratory, we have concluded that the Sickledex test is a version of the Itano solubility test which is predicated on the unique and extraordinary insolubility of deoxygenated hemoglobin S in phosphate buffer systems. We show that other non-S sickling hemoglobins and also unstable hemoglobins may and can give false positive Sickledex tests. We have modified the Sickledex test by the reversal of a positive test in the presence of urea when the specimen is S hemoglobin. If the test specimen is a non-S sickling hemoglobin the positive test will remain positive in the presence of urea. This differential property of the clearance of turbidity (the disbursal of the nematic liquid crystal system of sickled S hemoglobin) with urea under the conditions of this test is shown to occur only in the presence of unique, specific hydrophobic bonds between interacting tetramers, a condition which Murayama has shown to be essential to the sickling event in S hemoglobin. The modified Sickledex tube test as herein described will thus identify specifically not only S hemoglobin but also non-S sickling hemoglobins as a second category of hemoglobinopathies.

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MODIFIED SICKLEDEX TUBE TEST: A SPECIFIC TEST FOR S HEMOGLOBIN*

INTRODUCTION

The Sickledex tube test (1-4), a convenient and reliable screening test for S hemoglobin (Fig. 1), has been modified by us in a simple manner such that a "positive" screening test may easily be converted to a "positive" specific test for S hemoglobin. Furthermore, theoretical considerations indicate that the modification will discriminate for the presence of non-S sickling hemoglobins as well.

TECHNIQUE OF MODIFIED SICKLEDEX TUBE TEST

1. Materials:

a. Sickledex working test solution manufactured by Ortho Diagnostics, Raritan, New Jersey.

b. Urea-Sickledex working test solution. This reagent is prepared by dissolving a quantity of dry urea in the usual Sickledex working test solution to make a final concentration of 3 M with respect to urea.

2. Procedure:

a. Perform the standard Sickledex test in accordance with the directions of the manufacturer. When a "positive" Sickledex is noted, repeat the test, using the urea-Sickledex working test solution.

3. Interpretation:

a. When a "positive" Sickledex test changes to "negative" in the urea-Sickledex working test solution, either hemoglobin S or its structural variant, C (Harlem), is present.

b. When a "positive" Sickledex test REMAINS "positive" in the urea-Sickledex working test solution, a non-S sickling hemoglobin is present such as Bart's, Alexandra, C (Georgetown), or undiscovered types. Hemoglobin I, another non-S sickling hemoglobin, will give a negative standard Sickledex test.

NOTE: If the conversion of a "positive" to a "negative" Sickledex test in the presence of 3 M urea is going to occur, it will be observed almost always within 2 min. However, the observation period for such possible conversions should extend for 10 min.

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DATA

Our data is summarized in Table 1.

TESTS IN CURRENT USE FOR THE DETECTION OF S HEMOGLOBIN

Electrophoresis: At pH 8.6, the following abnormal hemoglobins migrate like hemoglobin S: D (5), Flatbush (6), Zurich (7), Stanleyville-II (8-10), Sealy (11), (Sinai, Hasharon (12,13)), P (13), Etobicoke (14), Sabine (15), Gun Hill (16), Shimonoseki (17), Kokura (17), Umi (17), L Ferrara (18), Leiden (19), Lepore (20), Memphis/S (21,22), and G (Coushatta) (23). The following hemoglobins cannot be distinguished electrophoretically from hemoglobin S under other specified conditions: Russ (24, 25), Shimonoseki (17), Koln (26), G (Philadelphia) (27), G (Port Arthur) (28), G (Galveston) (28), G (Texas) (28), Alexandra (29,30), and Zurich (31). Thus, electrophoresis is not a reliable technique in and of itself for the specific identification.

Sickling Tests: At least seven human hemoglobins are known to sickle (32). These are: S (33), C (Harlem) (34-36), C (Georgetown) (37,38), I (39-42), Bart's (43,44), Alexandra (45), and Memphis/S (21,22). When hemoglobins from animal origins are taken into account, additional sickling molecular species of hemoglobins are known (46): deer, sheep, goat, mongoose, raccoon, hamster, and squirrel. Schneider et al (47) have furthermore discussed the nonspecificity and pitfalls of the sodium metabisulfite ($Na_2S_2O_5$) sickling test. Finally, under certain conditions, Isaacs (48,49) has shown that virtually any molecular species of hemoglobin can be made to sickle. Thus, the demonstration of sickling alone is not a specific identification for hemoglobin S.

Differential Solubility Tests: The work of Perutz et al (50,51) contributed to the development of the Itano differential solubility test to distinguish between S and D hemoglobin (5,52). Itano found that deoxygenated S hemoglobin was far less soluble in a 2.3 M phosphate buffer system than were other hemoglobins known in 1953. Since that time, several new hemoglobinopathies have either been demonstrated to have, or are believed to have, low solubilities in aqueous systems. These include King's County (53), Stanleyville-II (8-10), and C (Harlem) (34-36). In addition, those hemoglobins listed under sickling tests above can well be expected to have low solubilities, either identical with, or similar to, hemoglobin S on theoretical grounds.

Yakulis and Heller (54) presented an elegant modification of the Itano solubility test known as the Slide Elution test, but this procedure has the same limitations of specificity as the Itano solubility test itself. In fact, Heller and Yakulis (55) reported subsequently that hemoglobin H, a nonstable Heinz body-forming hemoglobinopathy, yielded a false positive Slide Elution test. They also suggested that other unstable hemoglobins may behave similarly. Other unstable Heinz body-forming hemoglobins may have low solubilities and thus may present false positive

Slide Elution test (56). This flaw is also true of any other test based solely on a solubility parameter such as that reported by Ratanaubol et al (57).

The Sickledex test was originally introduced as specific for hemoglobin S (1). Nalbandian and Kessler (32) first noted that the Sickledex test on theoretical grounds could not be considered specific and was best regarded by them as a convenient screening procedure for the detection of S hemoglobin. They predicted on theoretical grounds that non-S sickling hemoglobinopathies would give false positive Sickledex tests. Subsequently, it has been learned that Bart's, but not I gives a false positive Sickledex test, confirming their contention in part (58).

Murayama Test: Very recently Nalbandian et al (59-67) have published the Murayama test, the first specific test for S hemoglobin predicated on unique features of molecular pathology of S hemoglobin. This test, though specific for the molecular pathology of S hemoglobin and relatively simple to perform, is not convenient for mass screening programs.

Principle of the Sickledex Test: Neither the principle nor the components of the Sickledex test (Fig. 1) have been disclosed. We believe that the Sickledex tube test is either identical with, or derived from, the original Itano solubility test on the basis of our unpublished experiments and data. We, therefore, believe that the working solution of Sickledex test contains approximately 2.3 M of phosphate, a deoxygenating agent, probably sodium hydrosulfite ($Na_2S_2O_4$), and a lysing agent, presumably saponin. If, into such a solution S hemoglobin erythrocytes are introduced, the erythrocytes will lyse, the hemoglobin will deoxygenate and polymerize forming microfilaments and microcables, i.e., the hemoglobin will "sickle." In the Sickledex solution, hemoglobin S thus forms a nematic liquid crystal system and thereby acquires some remarkable properties: (1) birefringence, (2) circular dichroism, (3) paramagnetism, and (4) light scattering. A positive and a negative Sickledex test are shown in Figure 1. The Sickledex test is essentially a solubility test for deoxygenated hemoglobin and so perforce is nonspecific.

CONVERSION OF A POSITIVE SCREENING SICKLEDEX TEST TO A POSITIVE SPECIFIC TEST FOR S HEMOGLOBIN

There is a molecular basis for our modification of the Sickledex test which achieves specificity for the identification of S hemoglobin. Murayama's recently modified hypothesis for the molecular mechanism of sickling clearly implicates the presence of unique hydrophobic bonds between interacting tetramers of hemoglobin S (64-67). Hydrophobic bonds in the recent physical chemistry literature are regarded as the most important single chemical bond for the maintenance of tertiary structures of proteins in aqueous systems (64-67). Very recently it has been discovered that urea, in addition to its well-known capability of breaking hydrogen bonds, is able to break hydrophobic bonds as well (64-67). Nalbandian et al have shown optical and electron microscopic evidence

for the reversal of sickling by urea in invert sugar (without hemolysis) (68-73). Furthermore, Nalbandian et al have reported two cases in which a sickle cell crisis was aborted by the use of urea in invert sugar (71). Hence, it was logical to use urea in the Sickledex test to determine whether specificity could be achieved with the Sickledex tube test by a chemical attack on the hydrophobic bonds essential to the sickling event.

On the basis of experiments and data not reported here, we determined that a 3 M urea final concentration at room temperature in a Sickledex working test solution (S hemoglobin present) would convert a positive screening test to a negative test almost always within 2 min (Fig. 2). A more extensive discussion of the molecular basis and the specificity for the reversal of sickling by urea is offered by Nalbandian et al elsewhere (70,71,73). Briefly, we believe that under the conditions of the Sickledex test, the S hemoglobin tetramer is deoxygenated. This produces a beta chain shift laterally of 6.9 Å relative to the central axis of molecular symmetry of the interacting S hemoglobin tetramers as shown by Muirhead and Perutz (74,75). Under such steric conditions, according to the Murayama hypothesis, the reciprocal steric combining sites between amino acid residues on the beta globin chains of one tetramer and those on the alpha globin chains of the other interacting tetramer are within critical distances sufficiently to form hydrophobic bonds. This reversal of a positive to a negative test occurs because of the action of urea in breaking hydrophobic bonds, inducing alterations in the tertiary structure and hence sterically hindering molecular stacking and sickling. This phenomenon, we believe, is specific only for S hemoglobin, since only hemoglobin S or the structural variant, C (Harlem), is known to sickle on the basis of hydrophobic bonds (64,66,67). Careful readings of the original sources of the cited references above dealing with non-S sickling hemoglobins will show that the molecular mechanisms are different for the different molecular species, with the single exception of hemoglobin C (Harlem) which is structurally identical to S hemoglobin in the N-terminal ends of the beta S globin chains (34-36).

A single specimen of hemoglobin I tested by us gave a negative Sickledex test. Thus, hemoglobin I cannot be identified either by the Sickledex test or by our modification.

Given a non-S sickling hemoglobin (except I or C (Harlem)), we expect on the theoretical grounds that a positive Sickledex test will remain positive after urea is added to the system. A simulated illustration of expected reactions is shown in Figure 3. This aspect of our modification remains to be confirmed or corrected experimentally by those who have access to these rare non-S sickling hemoglobins.

CONCLUSION

We contend that when a positive Sickledex screening test is treated with 3 M urea and converts from a positive test to a negative test, then a positive screening test has been converted into a positive specific

test for the molecular lesion of S hemoglobin. Since this reversal phenomenon can only occur when hydrophobic bonds unique to S hemoglobin or its structural variant, C (Harlem), are involved, it will be seen that the use of the Sickledex tube test in conjunction with this modification of the use of urea will be an excellent method for the detection of not only S hemoglobin but also non-S sickling hemoglobins as well.

The non-S sickling hemoglobins cited above, and not otherwise qualified, sickle on the basis of molecular mechanisms which do not involve hydrophobic bonds as does S hemoglobin. Hence such non-S sickling hemoglobins (such as Bart's) will give, we postulate, a false positive Sickledex test. In the presence of urea in such a system the conversion of a positive to a negative Sickledex test will not occur, since no hydrophobic bonds are responsbile for sickling. Thus, non-S sickling hemoglobins will be expected to give a positive Sickledex test which remains positive after the addition of urea. Hemoglobin S or its structural variant C (Harlem) will convert from a positive to a negative Sickledex test. Other hemoglobins will give a negative Sickledex test initially.

Our group welcomes samples of these rare non-S sickling hemoglobins so that we may further confirm or correct our theories in this regard.

A more comprehensive overview of the molecular aspects of sickle cell hemoglobin and applications to problems of laboratory diagnosis has been published by us elsewhere (76).

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Positive **Negative**

Fig. 1. A positive and a negative Sickledex test are shown. Note that in the negative Sickledex test there is no interference with the transmission of light through the working solution and the lines behind the test tube are clearly visible. In the positive test note that there is sufficient turbidity and interference with the transmission of light that the lines behind the test tube are totally obscured. Under the conditions of the test, when hemoglobin S [or its structural variant, hemoglobin C (Harlem)] is present, a nematic liquid crystal system forms. The significance of this physical-chemical state and the principle of the Sickledex test are discussed in the text.

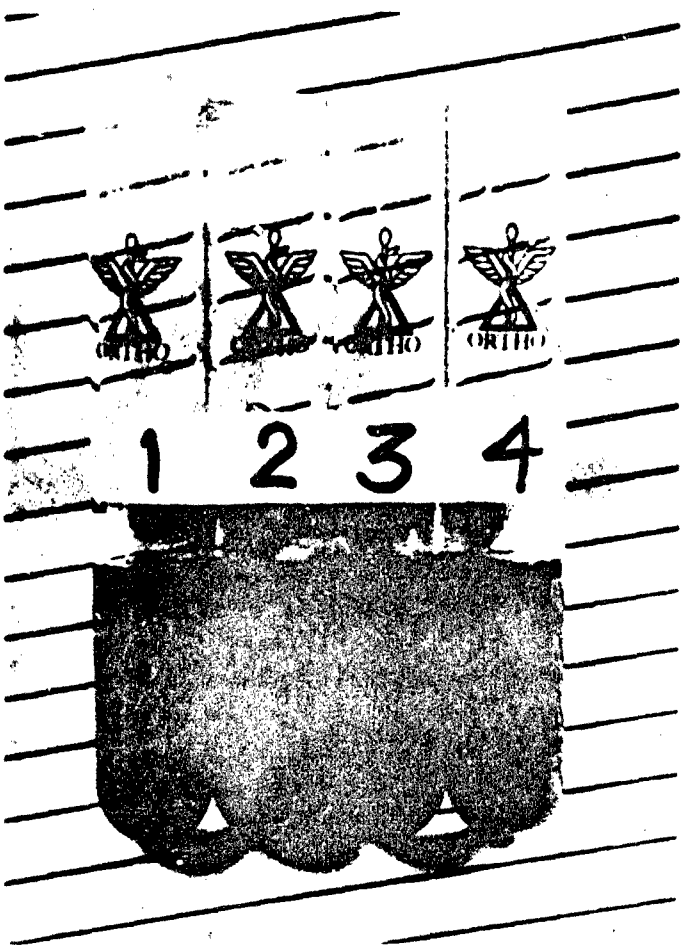

Fig. 2. Shown in this figure are controls and test results of our modified Sickledex tube test. In tube #1, hemoglobin A, in appropriate quantities, has been mixed with the Sickledex working test solution (the usual Sickledex test). In tube #2, hemoglobin A has been mixed with a Sickledex working solution which also contains a 3 M concentration of urea. Tube #3 contains an appropriate quantity of hemoglobin S in the Sickledex working test solution (the usual Sickledex test). In tube #4, hemoglobin S has been mixed with the Sickledex working test solution which additionally contains a 3 M concentration of urea. Note that in tube #3 a positive screening Sickledex test has been converted in the presence of urea to a negative specific test for hemoglobin S [or C (Harlem)] as shown in tube #4. Comparison of tubes #1 and #2 shows that urea does not adversely affect the standard Sickledex test when it is negative (see text).

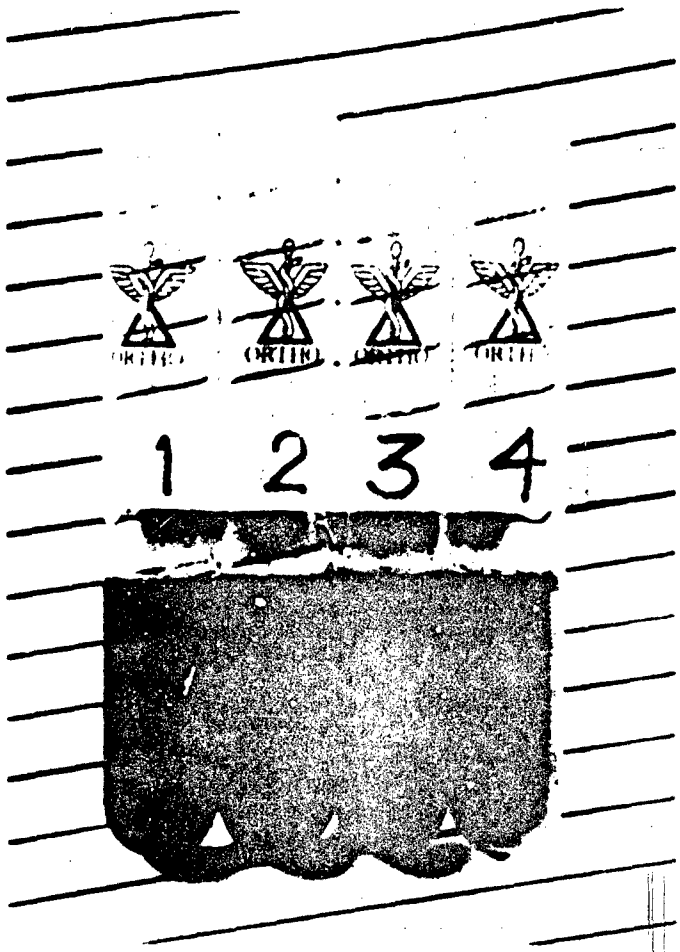

Fig. 3. This figure is a SIMULATED representation of the expected test results with the modified Sickledex tube test technique when a non-S sickling hemoglobinopathy [other than I and C (Harlem)] is encountered. In tube #1, hemoglobin A is mixed with the Sickledex working test solution (the standard Sickledex test). In tube #2, hemoglobin A is mixed with the Sickledex working test solution to which has been added a 3 M concentration of urea. In tube #3, we have SIMULATED the expected results when a non-S sickling hemoglobin, as qualified above, is mixed with a Sickledex working solution. A positive Sickledex test is expected. In tube #4, we have SIMULATED the test result when such a hemoglobin has been mixed with a Sickledex working solution to which a 3 M concentration of urea has been added. Thus, it is expected that when such non-S sickling hemoglobinopathies give a positive standard Sickledex test, the test will remain positive in the presence of a urea-Sickledex test solution (tube #4) since the nematic liquid crystal system is mediated by a molecular mechanism other than the hydrophobic bond (see text).

TABLE 1

Tests of Hemoglobin Identification

Test	Hemoglobin Type					
	SS	AS	CS	AC	AD	AA
Modified Sickledex	8 Pos	22 Pos	3 Pos	6 Neg	1 Neg	264 Neg
$Na_2S_2O_5$ Sickling	8 Pos	22 Pos	3 Pos	6 Neg	1 Neg	264 Neg
Murayama	6* Pos	8** Pos	1* Pos			
Electrophoretic mobility pH 8.4	8 same as S	22 same as A&S	3 same as C&S	6 same as A&C	1 same as S	264 same as A

*Not done in 2 cases.

**Not done in 14 cases.

A total of 304 different human blood specimens was tested. Forty of these represented hemoglobinopathies of S or C; 264 of the specimens were of A hemoglobin type and represent the controls. Confirmation of the hemoglobin type was established by the methods listed. Where quantities of blood were insufficient, the Murayama test was not done. The results are in support of the thesis of this report. These identical blood specimens were used simultaneously in an automated version of this modification. The results of the automated technique have been reported by Henry et al in USAMRL Report No. 898 (77).

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13. ABSTRACT

The Sickledex test is a screening method for the detection of S hemoglobin. However, the principle of this test has never been disclosed by the developers of the test. On the basis of analytical studies conducted in the Blodgett Memorial Hospital laboratory, we have concluded that the Sickledex test is a version of the Itano solubility test which is predicated on the unique and extraordinary insolubility of deoxygenated hemoglobin S in phosphate buffer systems. We show that other non-S sickling hemoglobins and also unstable hemoglobins may d can give false positive Sickledex tests. We have modified the Sickledex test by the reversal of a positive test in the presence of urea when the specimen is S hemoglobin. If the test specimen is a non-S sickling hemoglobin the positive test will remain positive in the presence of urea. This differential property of the clearance of turbidity (the disbursal of the nematic liquid crystal system of sickled S hemoglobin) with urea under the conditions of this test is shown to occur only in the presence of unique, specific hydrophobic bonds between interacting tetramers, a condition which Murayama has shown to be essential to the sickling event in S hemoglobin. The modified Sickledex tube test as herein described will thus identify specifically not only S hemoglobin but also non-S sickling hemoglobins as a second category of hemoglobinopathies.

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Itano solubility test						
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